IMPROVED DISCRIMINATION OF BACTERIAL SPORE SPECIES WITH FT-IR SPECTROSCOPY BY PRETREATMENT WITH AUTOCLAVING

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ABSTRACT

Fourier transform infrared reflectance microspectroscopy was applied to the identification of bacterial endospores. Mid-infrared vibrational spectra were collected following minimal sample preparation from five species of bacterial endospores: *Bacillus cereus*, *Bacillus globigii*, *Bacillus megaterium*, *Bacillus subtilis*, and *Clostridium perfringens*. Discrimination among these species is enhanced when the endospores are autoclaved prior to data collection. This pre-measurement autoclaving technique is attractive by itself as it places the samples obtained, even false positives, into a safe state for handling. Speciation of these microorganisms can be accomplished by multivariate techniques such as principal component analysis (PCA) and soft independent method of class analogy (SIMCA).

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INTRODUCTION

A method for the rapid, and safe detection and classification of bacterial endospores is extremely important due to the pathogenic properties some possess. For example, *Bacillus cereus* is known to cause food poisoning. Some potentially pathogenic microorganisms, specifically from the genera *Bacillus* and *Clostridium*, can sporulate at times of insufficient nutrients. The resulting endospores are highly resistant to environments that kill vegetative bacteria, allowing the endospores to remain dormant until the right conditions are met for germination and subsequent normal growth. It is for this, and its pathogenic properties, that *Bacillus anthracis* is one bacterial microorganism that has been associated with biological warfare. [1]

Presently there exist several methods for the detection and discrimination of microorganisms. These include, but are not limited to, amplification of nucleic acids by polymerase chain reaction (PCR),[2] fluorescence spectroscopy, [3-11] and mass spectrometry.[12-17] Although these techniques exist there is still a need for rapid and complementary techniques to screen for these bacterial microorganisms that present a public heath problem.

Fourier transform infrared spectroscopy (FTIR) has been explored by several research groups as a means to classify, discriminate and identify species of bacteria, a detailed review of which has been reported.[18] This work was pioneered by Naumann et al., and was facilitated by improvements in the sensitivities of FT-IR instruments and computing in the 1990's.[19-21] These authors, with their extensive studies of different species and strains of bacterial cells, have shown that the mid-infrared spectral absorptions of whole bacteria are highly specific fingerprints, and that multivariate techniques for data analysis permit characterization of bacteria down to the subspecies level. Furthermore, FT-IR spectroscopy has also been used to identify cellular components and bio-markers of bacteria and bacterial endospores.[13, 22-24]

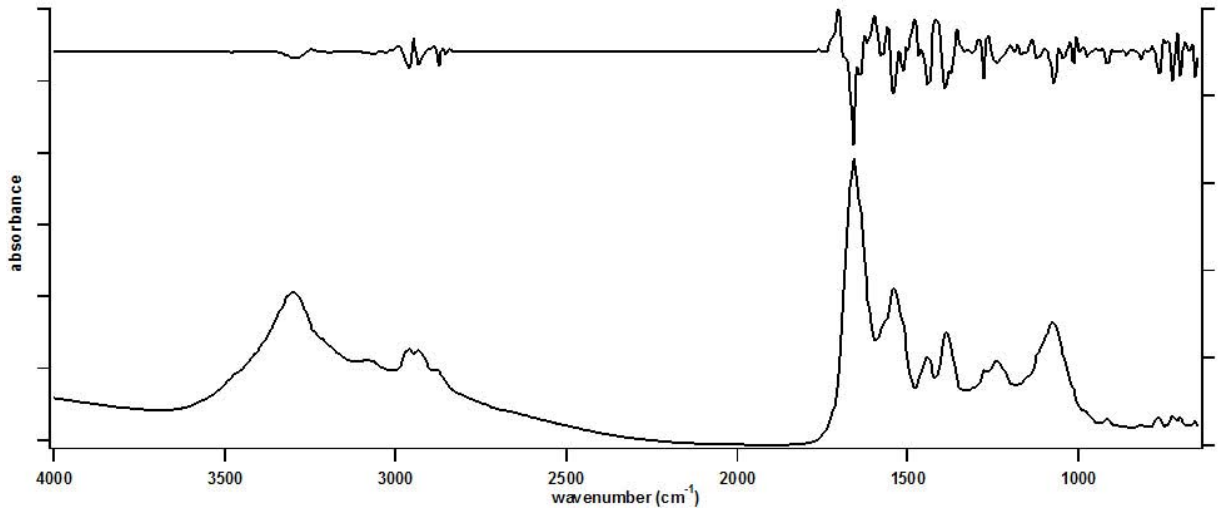

Figure 1. Representative mid-infrared absorbance spectrum and its calculated and 2nd derivative spectrum of a *B. cereus* endospore sample obtained at 8 cm^{-1} resolution.

It is highly desirable to search for simple preparation steps which can be automated and can enhance species differences in IR spectra. One routine technique used in health care and other institutions, to produce sterile materials and instruments, is the autoclaving process. Autoclaving provides sterilization by employing pressurized steam (15 psi) to obtain a saturation temperature of 121 °C, and it is used routinely to sterilize instruments in medical and research applications. If done correctly, autoclaving is an

excellent and dependable method for sterilization. The heat of autoclaving denatures and damages cellular macromolecules including proteins, cytoplasmic membranes, and nucleic acids. Due to the resistive properties of endospores, they themselves are frequently used in validation procedures to determine the effectiveness of autoclaves.

In a recent report from this laboratory,[25] our analysis of endospores of *Bacillus* and *Clostridium* species indicate that the two mid-infrared spectral regions, the amide I band (1500-1700 cm^{-1}) and C-H regions (2700-3500 cm^{-1}), are of interest in classification methods. Figure 1 illustrates a representative absorbance spectrum obtained in these studies, along with its calculated 2nd derivative spectrum. The amide I band (primarily a C=O stretch) yielded species separation utilizing multivariate techniques in untreated samples only. However, species separation exists in the C-H region in both reference and autoclaved samples.

The aim of this study is to evaluate the potential of FT-IR reflectance microspectroscopy in the development of a stepwise detection and classification scheme for bacterial endospores. We report the multivariate analysis of the mid-infrared reflectance spectral data obtained from *B. cereus*, *B. globigii*, *B. megaterium*, *B. subtilis*, and *C. perfringens* both prior to and following the autoclaving proceedure. This study indicates that in the multivariate methods used; PCA and SIMCA, autoclaving endospores allows for increased discrimination ability.

EXPERIMENTAL SECTION

Endopores of *Bacillus cereus* T, *Bacillus subtilis* strain PS832, a prototropic laboratory strain derived from strain 168 and *Bacillus megaterium* QM B1551 were prepared in liqiud SNB (*B. cereus* and *B. megaterium*) or 2xSG medium at 24 °C (*B. subtilis*), 30 °C (*B. cereus* and *B. megaterium)*, and 44 °C (*B. subtilis*). Endopores of *Clostridium perfringens* NCTC 9268 were the gift of Hillel S. Levinson (formerly of the U. S. Army Natick Laboratories, Natick, MA). These endospore preparations were cleaned with numourous suspensions of water followed by centrifugation, until the preparations were free of (>95%) of sporulating cells, germinated spores or cell debris as determined by phase contrast microscopy. These endospore samples lyophilized and stored dry. [26, 27]

The fifth strain of bacterial spores used was *Bacillus globigii*, provided from the Dugway Proving Grounds (Dugway, UT), and were a typical sample of the milled preparation used for the field testing of instruments. Although *B. globigii* has been reclassified as a substrain of *Bacillus atrophaeus* typified genetically by ATCC 9372,[28] for the purposes of this study we refer to this strain as *B. globigii*. They were prepared under contract by fermentation in liquid media containing 10 gm of Peptone HCT (hydrolized protein digest of pork from Marcor Development, Carlstadt. NJ); 5gm of Amberex 1003 (yeast extract); 0.5 gm of $MgSO_4 \cdot 7H_20$; 0.13 gm of $MnSO_4 \cdot H_20$; 0.27 gm of $CaCl_2 \cdot 2H_20$; 1.0 gm of K_2HPO_4; 0.5 gm of Pluronic antifoam (BASF Corp., Mt. Olive, NJ); and 10 gm of Dextrose per liter of sterile water. The Dugway endospores were concentrated and kept refridgerated until spray dried.

In addition to cleaning of the bacterial endospores mentioned above, samples were re-suspended in water, rinsed, sonicated, and centrifuged, after which the supernatant was removed and discarded. This procedure was repeated twice to further clean the samples of extraneous materials. The resulting samples were re-suspended in water and divided into two portions, one of which was prepared for autoclaving, and the other set aside for use as a reference. Samples for autoclaving were transferred to an appropriate vented test tube and placed in a Market Forge Sterilmatic STM E autoclave for 20 minutes. One drop of each autoclaved and untreated sample was placed onto a gold microscope slide and allowed to dry in a 50 °C oven. A second set of endospore samples was prepared under the same conditions as above for validation of the method.

FT-IR reflectance spectra were collected with 8 cm^{-1} resolution from 650 - 4000 cm^{-1} using a Nexus 470 FTIR spectrometer coupled with a Continuμm® microscope, both obtained from Thermo Nicolet (Madison WI). The FT-IR microscope was fitted with a liquid nitrogen cooled MCT-A (mercury-cadium-tellurium) detector, and the area sampled by the adjustable rectangular aperture was set to 100 x 100 μm. A triangle apodization function was utilized while co-adding and averaging 32 interferograms. A reference spectrum was obtained prior to each sample spectrum by positioning the X-Y microscope stage to a region free of sample on the gold microscope slide. Thirty spectra of reference and autoclaved samples for each of the 6 endospore preparations under study were collected, for a total of 360 spectra. This collection of spectral data was repeated with the duplicate validation samples.

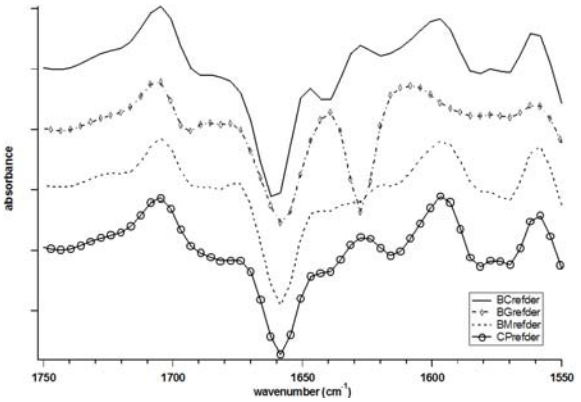

Figure 2. 2nd derivative spectra of untreated spores of *B. cereus* (BC), *B. globigii* (BG), *B. megaterium* (BM), *B. subtilis* (BS), and *C. perfringens* (CP), in the amide I region from 1550-1750. General peak assignments: 1650-1658 cm^{-1}: α-helix; 1620-1640 cm^{-1}: β-sheet; 1570-1580 cm^{-1} C-N str. of dipicolinic acid (DPA).[21, 29] Spectra have been offset for clarity.

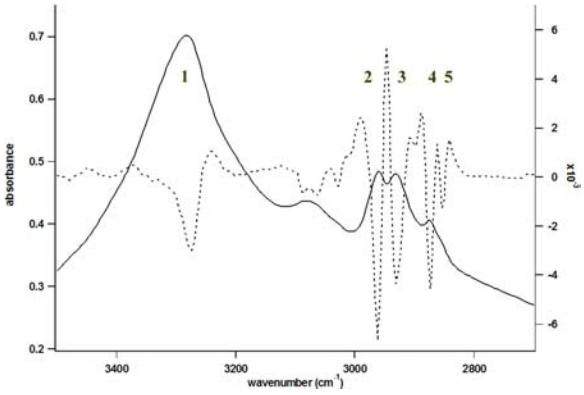

Figure 3. Absorbance (—) and 2nd derivative (---) spectra of *B. megaterium* endospores from 2700-3500 cm^{-1}. Peak assignments are as follows: **1**. N-H str. (amide A); **2**. C-H str. of $–CH_3$ (asym.); **3**. C-H str. of $>CH_2$ (asym.); **4**. C-H str. of $–CH_3$; **5**. C-H str. of $–CH_2$ associated with lipids and fatty acids.[20]

RESULTS AND DISCUSSION

One significant result of a previous study done by this group was the development of a technique to determine whether or not a sample of endospores was sterilized by autoclaving as monitored by FT-IR reflectance microspectroscopy.[30] The method was developed based on changes in the mid-infrared absorption spectra that occur in the amide I and II vibrations in the spectral range from 1500-1650 cm^{-1} (Figure 2). However, utilizing the absorbance spectra did not lend itself for species differentiation. Upon further analysis of the data, by calculating the second derivative spectra of the reference spores in the amide I band, there were differences noted among the 5 species studied, presumed to be result of the different conformations of the proteins that exist in the spores.[25] Furthermore, second derivative spectra of the autoclaved samples in the amide I region proved to be not useful in classification methods as the autoclaving technique caused the species to be undistinguishable. However, there was an observed separation in the spectra in principal component space in the second derivative spectra of both reference and autoclave data in the C-H region from 2700-3500 cm^{-1}. This region corresponds to the C-H stretching vibrations of lipids or fatty acids in the endospore coats, an example of which is illustrated in Figure 3.

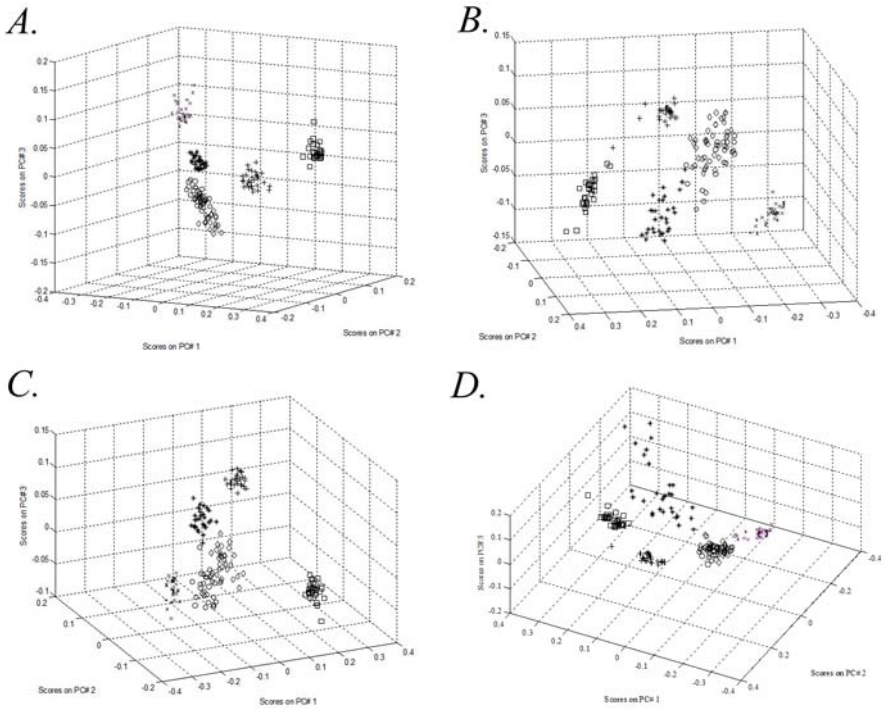

Figure 4. PCA scores plots for Set 1 autoclaved (A.), Set 2 autoclaved (B.), Set 1 reference (C.), and Set 1 reference (D.) of *B. cereus* (x), *B. globigii* (+), *B. megaterium* (*), *B. subtilis* (24°C (o)and 44°C (◊)), and *C. perfringens* (□) developed with 2nd derivative spectra from 2700-3500 cm^{-1}.

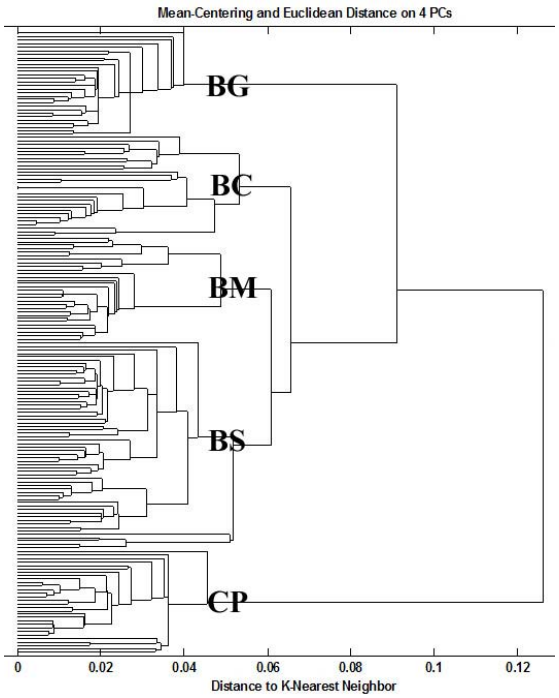

Figure 5. HCA dendogram of autoclaved (Set 1) endospores of *B. cereus* (BC), *B. globigii* (BG), *B. megaterium* (BM), *B. subtilis* (BS), and *C. perfringens* (CP) illustrating clustering of species.

The raw second derivative data were reduced to 2700-3500 cm^{-1}, vector normalized and mean centered and were used to develop the best PCA models. PCA scores plots of autoclaved and reference data were plotted against the first three principal components (Figure 4). Although there is separation in the five bacterial species in the reference data set (C) it is clear the autoclaved data set (A) yields better results. This was consistent with the results from the second experiment (B and D) respectively. Hierarchical cluster analysis (HCA) was also performed as a classification method. Although the results were inconclusive for determining whether autoclaved spores yielded better speciation in the C-H region from 2700-3500 cm^{-1}, the results in each case illustrated the natural clustering within the data by species. This is illustrated in Figure 5 for the autoclaved data (set 1) in which 4 principal components were kept and Euclidean distances calculated for use in developing the HCA model. Similar results were obtained for both autoclaved and reference samples when K-means or K- nearest neighbor algorithms were used, or Mahalanobis vs. Euclidean distance were calculated when 4 PCs were kept in each case. We note that the in both PCA and HCA models the *B. subtilis* samples grown at different temperatures (24 and 44 °C) cluster together, thus

temperature does not appear to effect speciation. We also note that the PCA clusters for *B. globigii* and *B. subtilis* are separated. This is particularly interesting since until recently these two phenotypically similar species were classified together.[31]

Set	Misclassifications	% Error
Set 1 autoclaved	1	1.7
Set 1 reference	22	36.7
Set 2 autoclaved	0	0
Set 2 reference	27	45

Table 1 SIMCA results summary for the four data sets based on FT-IR microspectroscopy in the C-H region from 2700-3500 cm^{-1}.

An attempt to quantify the discrimination of species utilized soft independent method of class analogy (SIMCA). First, 20 samples from each species were used to train the SIMCA model, while 10 were saved as unknowns, in a supervised method of classification. The results of this analysis show conclusively that the autoclaved endospores produce better results as illustrated in Table 1. Set 1 and set 2 reference data sets produced 22 (37% error) and 27 (45% error) misclassifications respectively, while set 1 and set 2 autoclaved data sets produced only 1 misclassification total (less than 2 % error for set 1).

CONCLUSIONS

A method has been developed for distinguishing species of spores of *Bacillus* and *Clostridium* species. This technique utilizes autoclaving, a technique common to those monitoring and studying such spores, which inherently reduces the risk associated with their handling. Any unknown sample or portion thereof, screened as a suspect pathogen, can be first autoclaved to reduce the risk of infection due to exposure, while monitoring the progress of autoclaving. Secondly, the unknown sample can be identified using this technique based on a set of known autoclaved samples. This classification utilizing autoclaving can be used to enhance FT-IR methods based on reference or untreated samples in the amide I region. Further analysis on a untreated portion of an unknown sample, or those false positives not accounted for in the model, could then be processed by more complicated and lengthy techniques to further classify the sample if required.

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